PORCINE UROPLAKIN II PROMOTER AND THE PRODUCTION METHOD OF USEFUL PROTEINS USING SAID PROMOTER

Technical Field

The present invention relates to a porcine uroplakin II gene promoter and a method for producing useful proteins using the same.

Background Art

In the medicinal field, as a method for maximizing the production of proteins such as EPO with high economic-value-added, a mass production method using cell culture technology has been mainly used. However, in this method, production cost is increased due to the use of animal blood as a culture medium, and expert knowledge is required for culture. Furthermore, since it is impossible to completely isolate a freshly produced EPO from an animal EPO contained in the culture medium, there is a problem in that a finally produced EPO has low purity and activity.

On the other hand, in a method for producing useful proteins using transgenic animals, a target protein is contained in body fluids secreted by the animals, so that a target protein is easily isolated and purified and maintains superior activity as compared to the existing cell-culture technology. For this reason, an interest in this method is being rapidly increased.

In the transgenic animal technology developed till now, mammary gland known to show high protein expression was mainly used as an organfor producing a target protein. However, the results of animal tests showed that it is ultimately impossible to produce several important target proteins, such as EPO, by expression in milk, due to expression in other tissues as well as the mammary gland.

Furthermore, since various proteins such as albumin are originally contained in milk at large amounts, the resulting target proteins are difficult to purify.

In an attempt to overcome such problems, a method for producing useful proteins using the bladder is recently proposed.

The bladder produces urine throughout animal's life regardless of the age and sex of animals, and the urine contains protein and fatty components at only a very small amount of 5-25 mg/l. Thus, the use of the bladder makes the isolation and purification of target proteins significantly easy.

However, the protein production efficiency of animals transformed with bladder-specific promoters developed up to now is still at a very low level.

Thus, there is an urgent need for the development of a promoter, which promotes the expression of a target protein at high efficiency.

Disclosure of Invention

Accordingly, it is an object of the present invention to isolate a porcine uroplakin II gene promoter, which promotes the bladder-specific expression of a target protein, and also to provide a method capable of producing useful proteins at large amounts using this promoter.

In one embodiment, the present invention provides a porcine uroplakin II gene promoter.

The porcine uroplakin II promoter preferably has a base sequence of SEQ ID NO: 1:

[SEQ ID NO: 1]:

gggctaggagtggaatcagagctggcctatgccacagcaacgcagaatccaaaccacatctccgacctaca
ccagaccgtcaccataacacaggatccttaacccactgagcaaggtcagggatcaaacccaaatcctcatggatactagt

cgggttcttaacccgctgagccacagtgggcactcctgtttttgtttgtgtcttcgttttttggctgcatctgcagcatacagaa

gttcctgggttaaggattgaacccatgccacagcagcaacccgagccacagcagtgacaacagcctgatccttaactgct

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ctcacttgcctcaggaaaccacagcttgccagcacccagcccagcaccagcccagct

Moreover, the porcine uroplakin II promoter of the present invention may be one selected from functional equivalents which have one or more disruption, deletion, insertion, point, substitution, nonsense, misense, polymorphism or rearrangement mutation occurred in the base sequence of SEQ ID NO: 1.

In another embodiment, the present invention provides an expression vector containing all or parts of the promoter.

The expression vector of the present invention preferably contains the promoter and also a base sequence coding for a target protein at the 3'-end of the promoter.

In another embodiment, the present invention provides an animal transformed with a fertilized ovum into which the expression vector was introduced.

In still another embodiment, the present invention provides a method for the mass production of useful proteins, which comprises collecting urine from the transgenic animal, and isolating and purifying the target proteins expressed in the urine.

The promoter of the present invention is located at the 5'-end of a porcine uroplakin II gene and regulates the expression of the porcine uroplakin II gene.

The promoter of the present invention can be isolated by screening a porcine genomic library in the following manner.

In order to obtain parts of the base sequence of the porcine uroplakin II gene to be used as a screening probe, the uroplakin II base sequences of other animals with known base sequences are compared to each other and a primer set (forward primer: SEQ ID NO: 2, and reverse primer: SEQ ID NO: 3) is constructed with reference to portions that are well conserved between the species. Then, RT-PCR is performed with the primer set, using the total RNA of the porcine bladder as a template.

After parts of the uroplakin II fragment are obtained through the RT-PCR reaction, a porcine genomic library is screened using the obtained parts as probes. As shown in FIG. 2, the probes used in the present invention are two probes consisting of probe A containing a portion of exons 2-5 of the uroplakin II gene, and probe B containing a portion of exons 1-2 of the uroplakin II gene.

As shown in FIG. 2, the library screening gives clones containing the uroplakin II gene or promoter. The base sequence of the promoter is finally determined by the comparison between the base sequences of the clones, thereby obtaining the complete base sequence of the porcine uroplakin II promoter.

The promoter thus obtained has a total size of 8847 bp, shows high G + C content, a characteristic of a housekeeping gene, in its base sequence, and contains various Sp1 elements, including AP2 and GATA boxes.

The promoter of the present invention specifically expresses a target protein only in bladder tissue among various porcine tissues. In the case of the porcine uroplakin II gene, it is expressed at 8-14% of total bladder cells, and actively propagated, particularly in an urothelial suprabasal cell, and shows high expression level in an umbrella cell being segmented.

Thus, since the promoter of the present invention induces the bladder-

specific expression of protein at high efficiency, the use of the inventive promoter allows the production of an expression vector that expresses a target protein of foreign origin in a bladder-specific manner.

In producing the expression vector of the present invention, the inventive promoter is inserted into the existing vector for protein expression, as a basic backbone, and a base sequence coding for a target protein is inserted into the 3'-end of the promoter, thereby producing the inventive expression vector.

The vector, which can be used as a basic backbone in the production of the inventive expression vector, may be a suitable vector selected from general expression vectors, and its examples include pBluescript SK vectors with various cloning sites, and a retroviral vector, such as pLNCX.

The expression vector of the present invention can express all proteins, which are used as an active ingredient of medical drugs, and examples of such proteins include erythropoietin (EPO), aldosterone, adreno-corticotropin, blood clotting factors, gonado-tropin, insulin, prolactin, and vasopressin.

If necessary, the expression vector of the present invention may additionally contain regulators, such as another promoter, an enhancer, a selective marker, a untranslated region (5'-UTR), 3'-UTR, a polyadenylation signal, a ribosome-binding sequence, a base sequence that can be inserted into a certain site of genome, and an intron, at its suitable locations.

The present invention provides expression vector pUP2/hEPO capable of expressing human EPO under the regulation of the porcine uroplakin II promoter (FIG. 3). The expression vector pUP2/hEPO is a preferred example of the expression vectors containing the uroplakin II promoter.

In the expression vector pUP2/hEPO of the present invention, a pBluescript

SK(-) vector is used as a basic backbone, and a human EPO-coding gene (Lin F. K. et al, Proc. Natl. Acad. Sci, USA, Cloning and expression of the human erythropoietin gene, 82:7580-7584, 1985; SEQ ID NO: 4) is fused to the 3' end of the inventive uroplakin II promoter. The expression vector pUP2/hEPO was deposited under the accession number KCTC 10352BP on October 17, 2002 with the Korean Collection for Type Cultures (KCTC), Korean Research Institute of Bioscience and Biotechnology.

If necessary, the expression vector pUP2/hEPO of the present invention may additionally contain a neomycin-resistant gene, an insulator, or a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), such that the establishment of a transgenic cell line can be easily performed, the expression level of a target protein can be maximized and the stability of expression of the target protein can be ensured.

The neomycin-resistant gene is a gene showing resistance to a G418 reagent used in cell line establishment, and can act as an efficient selective marker in the establishment of an animal cell line, which express protein under the regulation of the UPII promoter. The neomycin-resistant gene has a base sequence of SEQ ID NO: 5:

[SEQ ID NO: 5]

gcggccgcgcgcgtcaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaataca
ttcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtcctgaggcggaa
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agatccttttgataatctcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtccgatcg

The insulator is a factor promoting the effect of a regulator adjacent to the promoter and also promoting position-independent expression, and allows a target protein to be stably expressed under the regulation of the UPII promoter. The insulator has a base sequence of SEQ ID NO: 6

[SEQ ID NO: 6]:

tcgactctagagggacagccccccccccaaagcccccagggatgtaattacgtccctcccccgctaggggca
gcagcgagccgcccggggctccgctccggtccggcgctcccccccgcatccccgagccggcagcgtgcggggacag

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tggagagatacagctgggtcagtaggactgggacaggcagctggagaattgccatgtagatgttcatacaatcgtcaaat
catgaaggctggaaagcctccaagatccccaagaccaaccccaacccacccaccgtgcccactggccatgtccctcagt
gccacatccccacagttcttcatcacctccagggacggtgacccccccacctccgtgggcagctgtgccactgcagcac
cgctctttggagaaggtaaatcttgctaaatccagcccgaccctcccctggcacaacgtaaggccattatctctcatccaac
tccaggacggagtcagtgaggatggggctctagagggacagccccccccaaagcccccagggatgtaattacgtccc
tcccccgctaggggcagcagcgagccgcccggggctccgctccggtccggcgctcccccccgcatccccgagccggc
agcgtgcggggacagcccgggcacggggaaggtggcacgggatcgctttcctctgaacgcttctcgctgctctttgagc
ctgcagacacctgggggatacggggaaaaagctttaggctgaaagagagatttagaatgacagaatcatagaacggc
ctgggttgcaaaggagcacagtgctcatccagatccaacccccctgctatgtgcagggtcatcaaccagcagcccaggct
gcccagagccacatccagcctggccttgaatgcctgcagggatggggcatccacagcctccttgggcaacctgttcagt
gcgtcaccaccctctgggggaaaaactgcctcctcatatccaacccaaacctcccctgtctcagtgtaaagccattccccct
tgtcctatcaaggggagtttgctgtgacattgttggtctggggtgacacatgtttgccaattcagtgcatcacggagaggc
agatcttggggataaggaagtgcaggacagcatggacgtgggacatgcaggtgttgagggctctgggacactctccaa
gtcacagcgttcagaacagccttaaggataagaagataggatagaaggacaaagagcaagttaaaacccagcatggag
aggagcacaaaaaggccacagacactgctggtccctgtgtctgagcctgcatgtttgatggtgtctggatgcaagcagaa
ggggtccatgtccctcagtgccacatccccacagttcttcatcacctccagggacggtgacccccccacctccgtgggca

gctgtgccactgcagcaccgctctttggagaaggtaaatcttgctaaatccagcccgaccctcccctggcacaacgtaag gccattatctctcatccaactccaggaacggagtcagtgag

The WPRE is a regulator contributing to the stabilization of mRNA so as to increase the synthesis of a target protein, and allows the target protein to be expressed at large amounts under the regulation of the UPII promoter. The WPRE has a base sequence of SEQ ID NO: 7:

[SEQ ID NO: 7]

accaggttctgttcctgttaatcaacctctggattacaaaatttgtgaaagattgactggtattcttaactatgttgct ccttttacgctatgtggatacgctgctttaatgcctttgtatcatgctattgcttcccgtatggctttcattttctcctccttgtataa atcctggttgctgtctctttatgaggagttgtggcccgttgtcaggcaacgtggcgtggtgtgcactgtgtttgctgacgcaa ccccccactggttggggcattgccaccacctgtcagctcctttccgggactttcgctttcccctccctattgccacggcgga actcatcgccgcctgccttgcccgctgctggacaggggctcggctgttgggcactgacaattccgtggtgttgtcgggga agctgacgtcctttccatggctgctcgcctgtgttgccacctggattctgcgcgggacgtccttctgctacgtcccttcggcc ctcaatccagcggaccttccttcccgcggcctgctgccggctctgcggcctcttccgcgtcttcgccttcgccctcagacg agtcggatctccctttgggccgcctccccgcctgtttcgcctcgggctcctcgag

The present invention provides an I/pUP2/hEPO vector, a pUP2/hEPO(WPRE) vector and an I/pUP2/hEPO(WPRE) vector, as preferred examples of the expression vector which additionally contains the regulators.

Such vectors are produced by inserting the neomycin-resistant gene into the inventive pUP2/hEPO vector, and then either inserting the WPRE into the 3' end of the EPO gene or inserting the insulator into the 5' end of the UPII promoter.

Examples of an animal, which can be transformed with the expression vector of the present invention, include all animals that urinate, such as porcine, mouse, bovine, poultry, ovine and caprine animals.

A method for the production of a transgenic animal using the expression

vector of the present invention is conducted according to the conventional method. Namely, a fertilized ovum is collected from a healthy individual among animals to be transformed, and the inventive expression vector is introduced into the fertilized ovum. Then, a pseudopregnant mouse is obtained using a vasectomized mouse, and the fertilized ovum is implanted into the oviduct of the pseudopregnant mouse as a surrogate mother. Then, transformed individuals among the descendants obtained from the surrogate mother are screened.

Thereafter, urine is collected from the screened individuals confirmed to be transformed, and then a target protein is isolated and purified from the collected urine, thereby producing useful proteins.

In the inventive method for the production of useful proteins, the isolation and purification processes of urine can be performed by the conventional technique, such as filtration or chromatography.

The inventive transgenic animal produced as described above expresses a target protein in a bladder-specific manner, and expresses the target protein in urine at a far higher concentration than the existing method.

For example, a mouse transformed with the expression vector pUP2/hEPO of the present invention shows a high EPO expression level of 0.5-1 mg/ml. Although EPO is a protein that is difficult to express since it causes the early death of an embryo, the inventive animal shows at least 1,000 times higher EPO expression level than the expression level of protein in urine, which is obtained using the existing uroplakin promoter.

Furthermore, the protein produced from the inventive transgenic animal shows a superior physiological activity to that of the same kind of commercially available protein.

For example, EPO obtained from a mouse transformed with the expression vector pUP2/hEPO of the present invention maintains the survival rate of an EPO-dependent hepatocyte cell line at a higher level than that of commercially available EPO.

As a result, the promoter of the present invention, and the expression vector and transgenic animal using the promoter, can be advantageously used in the production field of useful proteins that have been difficult to produce at large amounts.

Brief Description of Drawings

FIG. 1 shows the structure of probes used in isolating a porcine uroplakin II promoter of the present invention, and clones isolated by the probe;

FIG. 2 shows the structure of expression vector pUP2/hEPO of the present invention;

FIG. 3 shows the bladder-specific expression of a porcine uroplakin II mRNA;

FIG. 4 shows the urothelium-specific expression of a porcine uroplakin II protein;

FIG. 5 shows the expression level of a porcine uroplakin II protein in bladder cells and the umbrella cell-specific expression of this protein;

FIG. 6 shows the bladder-specific expression of EPO mRNA in a mouse transformed with the expression vector pUP2/hEPO of the present invention;

FIG. 7 shows the expression of an EPO protein in a mouse transformed with the expression vector pUP2/hEPO of the present invention;

FIG. 8 shows the structure of the expression vector pUP2/hEPO of present

invention;

FIG. 9 shows the structure of the expression vector pUP2/hEPO of the present invention;

FIG. 10 shows the structure of the expression vector pUP2/hEPO of the present invention;

FIG. 11 shows the comparison between the EPO gene expression levels of the inventive expression vectors; and

FIG. 12 shows the comparison between the EPO protein expression levels of the inventive expression vectors.

Best Mode for Carrying Out the Invention

The present invention will hereinafter be described in further detail by examples. It should however be borne in mind that the present invention is not limited to or by the examples.

Example 1: Isolation of inventive porcine uroplakin II promoter

In order to isolate the porcine uroplakin II promoter of the present invention, the following test was performed.

1) Preparation of probe by RT-PCR (Reverse Transcriptase- Polymerase Chain Reaction)

Since the base sequence of a porcine uroplakin II gene is not yet known, mouse and bovine uroplakin II cDNAs whose base sequences had been known were compared to each other. With reference to portions that are highly conserved between the two species, a degenerate primer set for use in the amplification of the porcine uroplakin II cDNA was produced. The base sequences of the forward and

reverse primers are shown in SEQ ID NOS: 2 and 3, respectively.

Using the primer set, RT reaction was performed on the total RNA of the porcine bladder with a MuMLV reverse transcriptase, and the resulting cDNA was subjected to PCR using a Taq polymerase. The reading of the base sequence of the amplified DNA showed that the amplified DNA is parts of an uroplakin II gene. The amplified DNA was cloned with a pGEM T-easy vector.

In order to produce a probe to be used in the isolation of an uroplakin II promoter, 50 ng of the cloned DNA was boiled for 3 minutes, and cooled in ice to denature it. The denatured DNA was added to a reaction buffer containing primer, dNTP, [α-^{32}P]dCTP (3000 Ci/nmol, NEN), and then a Klenow fragment was added to the solution and reacted at 37 °C for 1 hour. The probes thus obtained consist of probe A comprising a portion of exons 2-5 of the uroplakin II gene and probe B comprising a portion of exons 1-2 of the uroplakin II gene (FIG. 1).

Then, the reaction solution was purified using a Sephadex G-50 column, thereby preparing ^{32}P-labeled DNA probe A and probe B for porcine uroplakin II promoter probing.

2) Library Screening

To isolate the porcine uroplakin II promoter, a porcine genomic library was screened. In this example, the porcine genomic library which had been inserted into a lambda Fix II phage vector (Stratagene) was used.

Host bacteria to be introduced with the library were prepared as follows.

5 ml LB medium containing 0.2% maltose was inoculated with one bacteria colony and cultured at 37 °C overnight. 1% of the culture medium was transferred into 50 ml of a fresh LB medium containing 0.2% maltose and cultured for 2.5 hours. When the absorbance at 600 nm reached about 0.5, the culture solution was

centrifuged at 2,500 rpm for 10 minutes. The resulting cell precipitates were suspended in 10 ml of sterilized magnesium sulfate solution to a final concentration of 1 x 10^{10} cells/ml, and stored at 4 °C until test.

For titration, the library was serially diluted in SM solution at various concentrations. A plate containing solid LB medium was warmed in a 37 °C incubator, and top agar was dissolved and put in a water bath kept at 48 °C. 10 μl of each of the phage solutions diluted at various concentrations was mixed with 100 μl of the above prepared host bacteria, and the host bacteria were infected with the phage at 37 °C.

The host bacteria infected with the phage were added to the top agar, well shaken, and then poured on the above-prepared LB medium. After 15 minutes, the plate was turned upside down and cultured in a 37 °C incubator overnight. On the medium of the plate cultured overnight, plaques indicating that the phage dissolved the host bacteria after reproducing library DNA in the host bacteria were formed, and for use in a subsequent step, the plate was cooled at 4 °C for at least one hour.

NC filters with serial numbers were provided, and the above-prepared library DNA plate was covered with the filters in such a manner that the middle portion of the filters was first contacted. The filters were pricked with a needle in a vertical direction to the filter so as to mark a position, and one minute later, the filters were carefully separated from the medium.

Each of the filters was successively immersed in denaturation solution, neutralization solution and 2 X SSC solution for one minute each solution, and then, placed in an oven at 80 °C for 2 hours such that the transferred library DNA was completely immobilized on the filter.

Each of the immobilized filters was floated on 2 X SSC solution to wet it,

and then prehybridized in a petri dish containing prehybridization solution with slow shaking at 68 °C for 1 hour. After the prehybridization, each of the filters was added with the probes prepared in the part 1) of Example 1, and hybridized with slow shaking at 68 °C for 18 hours. After the hybridization, the process of immersing the filters in 2 X SSC solution containing 0.1% SDS and washing the filters with shaking at 65 °C for 10 minutes was repeated two times. After the washing, the filters were dried in air and subjected to autoradiography.

By the comparison between the autoradiographic result and the plate, a plaque showing a positive sign was selected. The plaque was placed in 500 μl SM buffer solution, and one drop of chloroform was added to and well mixed with the solution, and the mixture was stored at 4 °C. Such a screening process was repeated three times, and clones showing a positive sign were finally obtained. DNA contained in each of the clones was purified using a Qiagen lambda mini kit.

The reading of the DNA base sequence was performed using an ABI 377 DNA sequencer (Applied Biosystem), the results of the sequence reading was processed using a CAP2 sequence assembly system, sequence comparison was performed using BLAST, SMART, PROSITE and the like, and motif analysis was conducted using a Clustal W program.

As a result, when the screening was performed with the probe A, clones A and B as shown in FIG. 1 were obtained. When the probe B was used in the screening, clones C and D as shown in FIG. 1 were obtained. Since each of such clones contained a porcine uroplakin II promoter or a structural gene at the 3' end, the comparison between the clones provided the complete base sequence of the porcine uroplakin II promoter.

The porcine uroplakin II promoter of the present invention has a total size of

8874 bp, and its base sequence is shown in SEQ ID NO: 1.

3) Examination of expression pattern of protein which is expressed under regulation of inventive promoter

In order to examine the expression pattern of a protein, which is expressed under the regulation of the inventive promoter, the expression of porcine uroplakin II was examined as follows.

3-1) Examination of bladder-specific expression of protein which is expressed under regulation of inventive promoter

In order to examine if a protein, which is expressed under the regulation of the inventive promoter, is expressed in a bladder-specific manner, Northern analysis was performed.

The porcine uroplakin II cDNA obtained in the part 2) of Example 1 was used as a probe, and at the same time, a probe for actin which is expressed in all tissues at a constant level was provided as a control group. In order to confirm the expression of the porcine uroplakin II mRNA in various porcine body tissues using the probes, total RNA for tissues including the bladder, the heart, the liver, the lungs, the womb and the spleen was subjected to electrophoresis as follows.

0.7 g of agarose was placed in a 250 ml Erlenmeyer flask, added with 58 ml of distilled water, completely dissolved in an electronic rang, and then cooled in a water bath kept at 60 °C. When the temperature of the agarose gel was adjusted to 60 °C, 7 ml of 10 x running buffer was carefully added with shaking, and 11.9 ml of formaldehyde was further added to prepare 1 × formaldehyde running gel solution. This solution was placed in a preset electrophoretic system and left to stand for about 20 minutes to produce gel.

6 μl RNA, 2.5 μl 10 × running buffer, 4 μl formaldehyde, and 12.5 μl

formamide were well mixed in a microcentrifuge tube, and heated at 65 °C for 5 minutes, and then cooled in ice. 2.5 μl gel-loading buffer was added to and well mixed with the sample, and loaded on gel, which had been pre-electrophoresed at 5 V for about 5 minutes. The resulting substance was electrophoresed in 1 x running buffer at 120 V/cm. After the electrophoresis, the gel was placed in 0.05 N sodium hydroxide solution for about 10 minutes, so as to partially cut RNA such that an efficiency in a subsequent transfer process is enhanced.

The gel was placed in 0.1 M Tris solution (pH 7.5) for 30 minutes, and in 20 x SSC solution (3M sodium chloride, 0.3M sodium-citrate, pH 7.3) for about 30 minutes, and then DNA was transferred to the gel using a positively charged membrane. For RNA immobilization, the transferred membrane was left to stand at 80 °C for 2 hours.

The membrane was placed in a vinyl bag in which hybridization solution was contained at a minimum volume at which the membrane can be completely submerged. Next, the bag was stored in a 68 °C shaking incubator for at least one hour. Then, the solution was drawn out, replaced by 15 ml of a hybridization solution containing the probe, and left to stand in 68 °C shaking incubator overnight.

After the hybridization, the membrane was washed with washing solution 1 (2 x SSC, 0.1% SDS) at room temperature for 30 minutes while replacing the washing solution, and then, washed with washing solution 2 (0.2 x SSC, 0.1% SDS) at 55 °C for 30 minutes while replacing the washing solution 2. After the membrane was completely dried at room temperature, it was subjected to autoradiography to examine if the porcine uroplakin II mRNA was expressed. The results are shown in FIG. 3.

As shown in FIG. 3a, the actin mRNA as an internal control group was

uniformly expressed in all the tissues. On the other hand, as shown in FIG. 3b, the uroplakin II mRNA which is expressed under the regulation of the inventive promoter was specifically expressed only in the porcine bladder (FIG. 3b).

As a result, it can be found that the promoter of the present invention expresses the protein in a bladder-specific manner.

3-2) Examination of urothelium-specific expression of protein which is expressed under the regulation of inventive promoter

Meanwhile, in order to examine if the protein which is expressed under the regulation of the inventive promoter is expressed in any cell of bladder tissue, immunohistochemical staining was conducted as follows.

A paraffin fragment of porcine bladder tissue was provided, and maintained in Histoclear solution for about 10 minutes to remove paraffin. The fragment was immersed in aqueous alcohol solution at gradually decreasing concentrations to dehydrate it, and then immersed in methanol containing 3% hydrogen peroxide and 0.05 N hydrochloric acid containing 0.1% pepsin, so as to prevent the fragment being nonspecifically stained.

The fragment was washed two times with TBS buffer (0.05 M Tris, pH 7.4, 0.85% sodium chloride) for 5 minutes, and then, subjected to blocking reaction in TBS which had been diluted with normal equine serum at a ratio of 1:5.

The blocked fragment was immersed overnight in TBS which had been diluted with a primary antibody at a ratio of 1:500. At this time, a polyclonal antibody which can specifically bind to the porcine uroplakin II protein was used as the primary antibody, and one drop of equine serum of an ABC kit was used as a negative control group.

The fragment which had been subjected to the primary antibody reaction was

washed two times with TBS for five minutes each time to remove an excess of the antibody, and then reacted with a biotin-attached secondary antibody for 30 minutes. Thereafter, the fragment was washed three times with TBS for 5 minutes, followed by reaction with an ABC reagent for 30 minutes. The fragment was washed with TBS again, rinsed with PBS containing 1% Triton-X 100 for 30 seconds, and then reacted with 0.05M Tris buffer (pH 7.6) containing 0.5% diaminobenzidine (DAB) and 0.01% hydrogen peroxide, to develop color.

After the color development reaction, the fragment was washed with water, mounted on an optical microscope, and observed for its developed portions. The results are shown in FIG. 4.

As shown in FIG. 4a, the control group did not show any positive sign. However, as shown in FIG. 4b, the reaction of the bladder tissue with the antibody to the uroplakin II protein showed that the promoter of the present invention regulated the uroplakin II protein such that the protein is specifically expressed only in the porcine urothelium, particularly in the cytoplasm of the suprabasal cell.

3-3) Examination of expression level of protein which is expressed under regulation of inventive promoter

Since the urothelium cell is known to have lower protein synthesis ability than that of mammary gland where the protein synthesis actively occurs, the actual expression level of the protein which is expressed under the regulation of the inventive promoter was examined by Laser scanning cytometry (hereinafter, referred to as 'LSC') in the following manner.

Porcine bladder tissue was split finely, added to DMEM/F12 medium (Gibco) containing 1mg/ml collagenase type I (Sigma), 0.51 mg/ml hyaluronidase (Sigma) and 50μg/ml gentamicin, and subjected to cleavage reaction at 37 °C for 1

hour.

After the resulting substance was washed with PBS, bulky masses were filtered out using a 60 μm nylon membrane (Milipore), and the suspended single cells were attached to a Lab-Tek chamber slide (Nunc) coated with 0.1% gelatin. The cells attached to the slide were washed with cold PBS and immobilized in cold methanol for 15 minutes, followed by treatment in 0.1% Triton-X 100 solution for 10 minutes.

The immobilized cells were blocked in 1% BSA-containing PBS solution for one hour and reacted with a 1:100 solution of the uroplakin II polyclonal antibody prepared in the part 3-2) of Example 1 at room temperature for 2 hours. After washing with PBS, the cells were reacted with a FITC-attached anti-mouse IgG secondary antibody (Cappel Laboratories). At this time, a group reacted with only the secondary antibody was also prepared as a negative control group.

After the cells were washed three times with PBS containing 0.1% Tween-20, they were stained with 50 μg/ml propidium iodide (PI) such that total cell number can be measured. Upon LSC analysis, fluorescent light was emitted with a 488 nm argon laser, fluorescent expression was observed using a 530 nm filter for FITC and a 570 nm filter for PI. The results are shown in FIG. 5. The results of analysis for the negative control group were shown in FIG. 5a, the results for analysis for the cell expressing the uroplakin II among the bladder cells were shown in FIG. 5b, and the results of analysis for the immune phenotype of the bladder cell expressing the uroplakin II were shown in FIG. 5c.

As shown in FIG. 5b, it was found that about 8-14% of the total bladder cells expressed the uroplakin II. As shown in FIG. 5c, it was found that most of the cells were umbrella cells being actively propagated and cleaved. Considering that

proteins in urine are generally at a very low level of 5-25 mg/l, the above-mentioned expression level of the uroplakin II is significantly high. Also, it is presumed that the use of bladder tissue allows proteins to be isolated and purified at a higher efficiency than the use of mammary gland tissue.

As a result, it can be found that the promoter of the present invention allows a target protein to be expressed in the bladder at excellent efficiency.

Example 2: Production of inventive expression vector pUP2/hEPO

Using the inventive promoter isolated in Example 1, a vector which expresses EPO under the regulation of this promoter was produced in the following manner.

A pBluescript SK(-) vector was selected as a basic backbone vector and inserted with the inventive promoter isolated in the part 2) of Example 1. Thereafter, a gene coding for human EPO (SEQ ID NO: 4) was inserted in the 3'-end of the promoter.

The resulting expression vector has a structure as shown in FIG. 2 and will express EPO under the regulation of the uroplakin II promoter of the present invention. This vector was termed "pUP2/hEPO" and deposited under the accession number KCTC 10352BP on October 17, 2002 with the Korean Collection for Type Cultures (KCTC), Korean Research Institute of Bioscience and Biotechnology.

Example 3: Production of fertilized ovum introduced with inventive expression vector pUP2/hEPO

A fertilized ovum introduced with the inventive expression vector

pUP2/hEPO produced in Example 2 was produced as follows.

1) Collection of fertilized ovum

At 3 days before collecting a fertilized ovum, PMSG was administered into the abdominal cavity of a female mouse, and at 5 p.m., after two days, the female mouse was administered with hCG and then cross-fertilized with a male mouse. In the morning the next day of the cross-fertilization, whether a plug had been produced in the female mouse or not was observed to examine if the female mouse became pregnant.

The mouse that has been confirmed to be pregnant was subjected to cervical vertebral dislocation, its abdomen open was cut with surgical scissors, and the connective tissue portion of the womb was separated. The portion between the oviduct and the womb was torn with a pincette, after which the portion between the ovary and the oviduct was cut with scissors. Then, the side of the womb in the portion torn with the pincette was cut and the oviduct was separated.

The separated oviduct was placed in M2 medium and put on an insulator board to prevent its temperature from being reduced. The oviduct ampulla was broken with a 1 ml needle under a microscope, and an embryo was collected. The collected embryo was placed in hyaluronidase solution that had been exposed to room temperature, and it was left to stand until a cumulus oophorus cell was detached.

The resulting solution was washed 2-3 times with M2 medium, centrifuged at 13,000 rpm for 5 minutes and washed 2-3 times with M2 medium again, and a normal fertilized ovum was screened. The screened fertilized ovum was washed 2-3 times in M16 medium coated with paraffin oil, and then, it was transferred and stored in a 37 °C incubator.

2) Microinjection of DNA into fertilized ovum

Using a micromanipulator, the expression vector pUP2/hEPO of the present invention was injected into the fertilized ovum collected as described above.

Example 4: Preparation of transgenic mouse which produces human EPO under regulation of inventive promoter

Using the fertilized ovum produced in Example 3, a transgenic mouse that produces human EPO under the regulation of the inventive promoter was produced in the following manner.

1) Preparation of vasectomized mouse

A vasectomized mouse to be used in making a surrogate mother pseudopregnant was prepared as follows.

A six-week-old ICR mouse was selected and anesthetized, and then the integument being about 1.5 cm apart above the pubic bone was incised about 1 cm along the pubic bone using a pincette and scissors. Standing to the right or left so as to prevent the incision opening being overlapped, the muscularis was incised, and a testicle descended to the scrotum was moved into the abdominal cavity. The testicle, the epididymis and the spermaduct, were separated from each other with a pincette, a membrane around the speraduct was separated with a pincette, and the spermaduct was cut with a heated pincette. After confirming that the spermaduct had been separated, the muscularis was sutured and the mouse was placed in a warmer until it came of the anesthetic.

2) Preparation of pseudopregnant mouse as surrogate mother

Before the test day, an ICR female mouse that had been confirmed as having estrus was cross-fertilized with the vasectomized mouse prepared in the part 1) of

Example 3. In the morning on the test day, whether a plug had been produced in the female mouse or not was observed to examine if the female mouse became pseudopregnant.

3) Embryo transfer into oviduct

The fertilized ova prepared in the part 2) of Example 2 were arranged in a line to a micropipette. The integument and muscularis of the anesthetized female mouse as a surrogate mother were slightly incised, and the ovary, the oviduct and the upper portion of the uterine horn were drawn out of the body using a pincette. The ovary was positioned in such a manner that a portion exposed through the ovarian cyst faces upward. Then, adipose tissue was inserted using a styptic device to fix the ovary. Under a stereoscopic microscope, the membrane of the ovarian cyst was removed, after which the oviduct and the ovary were drawn to look for fimbriae. Then, the front tip of the transplantation pipette was inserted 2-3 mm into the oviduct, and the fertilized ova were carefully implanted into the oviduct together with medium. Whether a first bubble, as a marker, of two bubbles in the pipette had been inserted into the oviduct was observed to examine if the fertilized ova were surely implanted into the oviduct.

Descendants were obtained from the surrogate mother mouse. To screen transgenic mice among them, Northern analysis was performed using the exons 1 and 2 of EPO as probes, and the analysis results showed that 12 of 76 mice were transformed.

The expression pattern of an EPO protein for the transgenic mice was examined and the results showed that the EPO protein was expressed in a bladder-specific manner.

Example 6: Production of human EPO from inventive transgenic mouse

1) Examination of expression level of EPO in urine of inventive transgenic mouse

To examine the expression level of EPO in the urine of the inventive transgenic mouse, urine was obtained from the transgenic mouse, and filtered and then subjected to HPLC analysis. To examine the protein components of each fraction, electrophoresis and Western analysis were performed and the results are shown in FIG. 7.

As is evident from the electrophoresis results in FIG. 7a and the Western analysis results in FIG. 7b, the urine obtained from the transgenic mouse of the present invention contained a high concentration of EPO.

The concentration of EPO in the urine was calculated to be an expression level of 0.5-1 mg/ml, which is remarkably higher than the protein expression level in milk that can be seen in the existing transgenic animal.

As a result, the transgenic animal prepared using the inventive promoter can produce a target protein in its urine at excellent efficiency.

2) Examination of physiological activity of EPO obtained from inventive transgenic mouse

To examine the physiological activity of EPO obtained from the transgenic mouse of the present invention, EPO obtained in the part 1) of Example 3 was added to EPO-dependent hepatocyte cells and cultured. At this time, a control group was added with commercially available EPO. At each of 24, 48 and 72 hours after the culturing, the survival rate of the cells was measured and the results are given in Table 1 below.

Table 1:

Culturing time	DMEM/F12 (%)	FBS	FBS + commercial EPO	FBS + inventive EPO
24	38.5 ± 6.8	54.9 ± 4.3	58.2 ± 6.6	72.1 ± 4.7
48	21.6 ± 7.4	39.9 ± 2.9	50.0 ± 2.4	60.4 ± 7.5
72	10.0 ± 4.6	20.8 ± 11.7	39.6 ± 3.8	53.9 ± 4.0

As shown in Table 1, it was observed that EPO isolated from the urine of the inventive transgenic mouse showed a higher physiological activity than the commercial EPO in all the time zones.

As a result, the use of the transgenic animal prepared using the promoter of the present invention allows the production of a protein having a far superior physiological activity to a protein that can be obtained by the existing method.

Example 6: Production of inventive expression vector containing regulators, and examination of its efficiency

1) Construction of expression vector containing regulators

In order to establish a vector system that can maximize EPO production under the regulation of the inventive UPII promoter, a selective marker and regulators were introduced into the pUP2/hEPO vector in the following manner to produce a series of improved vectors.

1-1) Construction of pUPII/hEPO-Neo vector

In order to insert an efficient selective marker into a vector in the establishment of a cell line that can express a protein under the regulation of the UPII promoter, a neomycin-resistant gene was introduced in the following manner so as to produce a pUP2/hEPO-Neo vector.

To obtain the neomycin-resistant gene, PCR reaction was performed using a

pEGFP-N1 vector (Clontech) as a template, and a forward primer (SEQ ID NO: 8) and a reverse primer (SEQ ID NO: 9).

5' - GCGGCCGCGCGCGTCAGGTGGCAC – 3' (SEQ ID NO: 8)

5' - CGATCGGACGCTCAGTGGAACGAAAACTC – 3' (SEQ ID NO: 9)

The resulting 1.9-kb PCR product was inserted into a pGEM T-easy vector and digested with a NotI restriction enzyme, to prepare the neomycin-resistant gene portion to be used in cloning.

The ampicillin-resistance gene site of the inventive pUP2/hEPO vector was removed by digestion with NotI and SalI restriction enzymes, to prepare a vector to be used in cloning.

The neomycin-resistant gene prepared as above described was cloned into to the vector, thereby producing the pUP2/hEPO-Neo vector where the neomycin-resistant gene was inserted into the existing pUP2/hEPO vector.

1-2) Construction of I/pUP2/hEPO vector

In order to obtain an expression vector which can stably express a protein under the regulation of the UPII promoter, an insulator gene was introduced into a pUP2/hEPO-Neo vector in the following manner so as to produce an I/pUP2/hEPO vector.

To obtain the insulator gene, PCR reaction was performed using a pBC1 vector (Invitrogen) containing a chicken B-globin insulator gene, as a template, and a forward primer (SEQ ID NO: 10) and a reverse primer (SEQ ID NO: 11). To increase PCR efficiency, two copies were amplified.

5' - TCGACTCTAGAGGGACAG – 3' (SEQ ID NO: 10)

5' - CTCACTGACTCCGTTCCT – 3' (SEQ ID NO: 11)

The resulting 2.4-kb PCR product was inserted into a pGEM T-easy vector

and digested with a NotI restriction enzyme, to prepare the insulator gene to be used in cloning.

The insulator gene prepared as described above and the vector of the above part 1-1) were coupled to each other by a NotI site, thereby producing the I/pUP2/hEPO vector (FIG. 8).

1-3) Construction of pUP2/hEPO (WPRE) vector

In order to obtain an expression vector that can express a protein at large amounts under the regulation of the UPII promoter, a WPRE gene was introduced into the pUP2/hEPO-Neo vector in the following so as to produce a pUP2/hEPO (WPRE) vector.

To clone the WPRE gene, PCR reaction was performed using a forward primer (SEQ ID NO: 12) and a reverse primer (SEQ ID NO: 13).

5' - ACCAGGTTCTGTTCCTGTTAATCAACCTC – 3' (SEQ ID NO: 12)

5' - CTCGAGGAGCCCGAGGCGAAACAGGCG – 3' (SEQ ID NO: 13)

The resulting 0.6-kb PCR product was inserted into the pGEM T-easy vector and then inserted into the NcoI restriction site of the pUP2/hEPO-Neo produced in the part 1-1) of this Example. The resulting vector was digested with a BspHI restriction enzyme, to prepare the WPRE gene to be used in cloning.

Meanwhile, the backside of the EPO gene of the inventive pUP2/hEPO vector was digested with an NcoI restriction enzyme, to prepare the vector to be used in cloning.

The WPRE gene prepared as described above was cloned into the vector, to produce the pUP2/hEPO (WPRE) vector (FIG. 9).

1-4) Construction of I/pUP2/hEPO (WPRE) vector

In order to produce an expression vector that can satisfy all the maximization

of expression level, the stabilization of expression and the establishment of an efficient cell line under the regulation of the UPII promoter, an I/pUP2/hEPO (WPRE) vector was produced in the following manner.

The insulator gene prepared in the part 1-2) of this Example was coupled with the vector of the part 1-3) of this example by a NotI site, thereby producing the I/pUP2/hEPO vector (FIG. 10).

2) Examination of efficiency of inventive expression vectors

The efficiency of the expression vectors produced in Example 6 was examined in the following manner.

2-1) PCR analysis for inventive expression vectors

To examine the expression level of the EPO gene, which is caused by inventive expression vectors, real-time PCR was performed as follows.

The four inventive expression vectors produced in Example 6 were introduced into bladder cell line RT4 using a transfection Kit (Effectene, Qiagen) and then subcultured to establish stable cell lines. Genomic DNA was extracted from each of the resulting cell lines and subjected to PCR to examine if the transfection was properly made.

To examine the expression level of the EPO gene, total RNA was extracted from the four cell lines and subjected to RT-PCR to amplify cDNA. The PCR was performed using the above cDNA as a template, and forward and reverse primers capable of amplifying the exon region of EPO.

To examine the expression level in each of the cell lines, this process was repeated three times using, as a control group, GAPDH that is a house keeping gene which is expressed in cells at a constant level. The test results were statistically processed using a SAS program and shown in FIG. 11 (pUP2 = pUP2/hEPO vector;

IUP2 = I/pUP2/hEPO vector; PW = pUP2/hEPO (WPRE) vector; and IW = I/pUP2/hEPO (WPRE) vector).

As shown in FIG. 11, the inventive expression vectors showed EPO gene expression level that is higher in the order of the pUP2/hEPO vector, the I/pUP2/hEPO vector, the pUP2/hEPO (WPRE) vector, and the I/pUP2/hEPO (WPRE) vector.

Particularly, the I/pUP2/hEPO (WPRE) vector which contains both the WPRE and the insulator showed about 50 times higher expression level than the pUP2/hEPO containing no separate regulators (FIG. 11b).

As a result, the inventive expression vectors, including the I/pUP2/hEPO (WPRE) vector, can be advantageously used for the production of EPO.

2-2) Western analysis for inventive expression vectors

To examine the expression level of EPO protein caused by the expression vectors of the present invention, Western analysis was performed as follows.

The cell lines which had been established by introducing the respective expression vectors of the present invention in the part 2-1 of Example 6 were placed in a lysis buffer containing NP-40 and sonicated to extract proteins from the cell lines.

Each 40 μl of the proteins were electrophoresed on SDS-PAGE gels, transferred to a PVDF membrane, and then treated with an EPO antibody to examine the expression level of an EPO protein. To quantify the expression level of the EPO protein, this process was repeated two times using an antibody for actin as a control group. The results were statistically processed with a SAS program and shown in FIG. 12 (pUP2 = pUP2/hEPO vector; IUP2 = I/pUP2/hEPO vector; PW = pUP2/hEPO (WPRE) vector, and IW = I/pUP2/hEPO (WPRE) vector).

As shown in FIG. 12, the expression vectors of the present invention showed the expression level of EPO protein, which is higher in the order of the pUP2/hEPO vector, the I/pUP2/hEPO vector, the pUP2/hEPO (WPRE) vector, and the I/pUP2/hEPO (WPRE) vector.

This result coincides with the results shown in the part 1) of Example 7.

As a result, the inventive expression vectors, including the I/pUP2/hEPO (WPRE) vector, can be advantageously used for the production of EPO.

Industrial Applicability

As described above, the promoter of the present invention induces the bladder-specific expression of a target protein, and expresses the target protein in urine at a far higher concentration than the existing method.

The animal, which was transformed with the expression vector consisting of the inventive promoter and the target protein being regulated by the promoter, secretes the target protein in urine at a far higher efficiency than that of the existing transgenic animal. Furthermore, the protein obtained from the transgenic animal of the present invention shows a superior physiological activity to the same kind of the existing protein.

As a result, the promoter of the present invention, and the expression vector and transgenic animal using this promoter, can be advantageously employed in the production field of useful proteins that are medicinally valuable.

PCT 03PP181

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0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.04.2003)
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1-1	page	8
1-2	line	16
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	Korean Collection for Type Cultures
1-3-2	Address of depositary Institution	52, Oun-dong, Yusong-Ku, Taejon 305-333, Republic of Korea
1-3-3	Date of deposit	17 October 2002 (17.10.2002)
1-3-4	Accession Number	KCTC 10352BP
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
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